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Variation in non-target traits in genetically modified hybrid aspens does not exceed natural variation

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ABSTRACT

Genetically modified hybrid aspens (*Populus tremula* L. *x P. tremuloides* Michx.), selected for increased growth under controlled conditions, have been grown in highly replicated field trials to evaluate how the target trait (growth) translated to natural conditions. Moreover, the variation was compared among genotypes of ecologically important non-target traits: number of shoots, bud set, pathogen infection, amount of insect herbivory, composition of the insect herbivore community and flower bud induction. This variation was compared with the variation in a population of randomly selected natural accessions of *P. tremula* grown in common garden trials, to estimate how the “unintended variation” present in transgenic trees, which in the future may be commercialized, compares with natural variation. The natural variation in the traits was found to be typically significantly greater. The data suggest that when authorities evaluate the potential risks associated with a field experiment or commercial introduction of transgenic trees, risk evaluation should focus on target traits and that unintentional variation in non-target traits is of less concern.

Introduction

Cultivation of transgenic plants is subject to strict regulation, particularly in Europe and elsewhere [1,2]. When the technique to produce transgenic plants, typically using the soil bacterium *Agrobacterium tumefaciens,* which transfers the T-DNA in its Ti-plasmid [3,4], was introduced in the 1980s, there were concerns that the technique could lead to unintended consequences, meaning that the technique as such had inherent risks. Such changes could occur through different mechanisms [5]. A gene in the T-DNA in the “new genome” could interact with the rest of the genome in a way that could not be predicted [6], e.g. an enzyme which in one organism converts substrate A to product B could, in theory, in another genetic transformation meet another substrate C that could be converted to product D. Alternatively, the function of a gene product may be unclear; there are many examples of proteins that have been found to be multifunctional or where the first assumptions of the function were incorrect [7]. There were also concerns about the lack of control of the point of insertion of the T-DNA and that interruption of a gene present at the site of insertion could cause unexpected changes, and in addition close physical interaction of two genes could potentially lead to effects that are hard to predict [6,8]. The randomness of the insertion site has been confirmed and is an asset: T-DNA KO [9] (T-DNA knockout) collections of *Arabidopsis thaliana* [10,11], *Oryza sativa* [12,13] and *Brachypodium distachyon* [14,15] have led to unprecedented discoveries concerning plant gene functions. Much effort has been spent to quantify “intentional” and “unintentional” variation between lines that have been generated through transformation [16]. Variation exists, and the scientific community has converged on a standard where several (at least three) independently transformed lines are necessary to confirm that a change in the phenotype is really caused by the insertion of the new genetic element [9,17].

The other trend in modern plant science is the exploration of natural

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Abbreviations: CV, coefficient of variation; GWAS, Genome-wide Association mapping; ’T89’, *Populus tremula x tremuloides* clone ‘T89’; WT, wild-type; SwAsp, the Swedish Aspen collection; “SwAsp 12”, one “type accession” from each of the 12 SwAsp populations; “SwAsp Halland”, ten SwAsp genotypes from Halland, local to the transgenic trials.

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variation. Again, research on *A. thaliana* has paved the way, but here other model systems have contributed greatly; the within-species variation of *A. thaliana* is abundant, but the lack of efficient outbreeding restricts gene flow and reduces the creation of new genetic combinations. Most other well-studied plant species have relatively low levels of genetic variation and as a consequence of domestication, gene flow has been severely restricted through breeding, so that both inbreeding and outbreeding agricultural crops typically have lower levels of genetic variation compared with their wild ancestors or undomesticated species [18].

Only a few decades ago it was widely assumed that each species had a set of genes, each of them having variations that evolution and breeders could act on to develop new combinations. However, even different accessions of *A. thaliana* often differ through the presence or absence of genes [19], while in highly variable species such as *Zea mays* [20] the fraction of the genome that is unique to only one variety is not much smaller than the fraction shared between two varieties. Analysis of genetic variation using Genome-wide Association mapping (GWAS) [21] is complicated by the fact that the unrelated individuals in a population have many other random genetic differences that may or may not affect the studied phenotype. As a consequence, GWAS populations have to be large to provide significant associations: a few hundred may only work in certain cases for traits controlled by only a few genes [22]. The reason that the numbers used in GWAS are at least two magnitudes larger than those to prove that transformation leads to a change of phenotype illustrates the dominance of natural variation over unintentional variation as a response to transformation with *A. tumefaciens* or in other words, natural variation outcompetes “unnatural variation”.

Studies on a larger scale that compare “unintentional variation” with natural variation between accessions have generally been performed using plants with relatively little genetic variation: crop plants or *A. thaliana*. The present work is with trees, in particular *Populus tremula* (European aspen) and, for transgenic work, *Populus tremula* L. *x P. tremuloides* Michx. (hybrid aspen). As genetic variation in trees is typically larger than variation in other species [23], it is not obvious how findings could be extrapolated to trees. One may argue that if natural variation is greater, the relative significance of unintended variation is decreased, but it is also possible that the amount of unintended variation could be greater in a highly heterozygous genome. Since transgenic trees are likely to be subject to approval processes within the European Union (EU) and risk evaluations for long-lived organisms like trees may be different from annual crops [24], there is a need to generate experimental data addressing this issue. The intention in this study was to compare variation in ecologically important traits in a collection of transgenic hybrid aspen lines to the variation among European aspen natural accessions. This work was made possible by access to collections of (1) transgenic hybrid aspen lines generated to understand the functions of aspen genes and (2) natural accessions of aspen. The latter have been randomly picked from the forest, cloned, and planted in common garden trials to study variation in the ecologically perhaps most important traits: growth, phenology, and tolerance to abiotic and biotic stresses.

Here the variation within two field experiments among the three independent transformation events for each gene in the hybrid aspen lines is examined. Next, the intentional variation in growth that is indicative of the productivity that the transformations intended to confer and unintended variation in non-target traits is examined. This is compared with a common garden experiment where the natural variation in traits among 12 natural aspen accessions from the Swedish Aspen (SwAsp) collection [25] could be directly compared to that in the same traits among the transgenic lines. The results from this common garden are also included with the variation in growth in a larger common garden for the SwAsp collection. The conclusions drawn from these experiments could be useful for evaluation of applications for field experiments and commercial introductions of transgenic trees.

Material and methods

Natural aspen accessions

For the natural accessions, the SwAsp (Swedish Aspen) collection of natural ecotypes sampled from around Sweden was used [25]. Two subsets of the SwAsp genotypes were used to compare to transgenics. One subset is the “SwAsp 12” set, one “type accession” from each of the 12 Swedish populations that is not, in any trait previously analysed [25–32], “atypical” for the entire collection (Supplementary material Table S1); this “SwAsp 12” set was planted in 2010 alongside the transgenic trial at Arlösa, Halland, southwestern Sweden, in three randomised blocks (Fig. 1, Table 1). A second sub-set of the SwAsp collection, “SwAsp Halland” is the population of ten SwAsp genotypes from Simlångsdalen, Halland, local to the transgenic trials, which due to its local origin exhibits less variability for growth phenotypes than the variation in the entire SwAsp collection that is drawn from ten degrees of latitude. The "SwAsp Halland" population was assessed at Ekebo, 90 km from Simlångsdalen (see Supplementary material, Methods).

Transgenotypes

Transgenic trees with up- or down-regulation (by RNAi) of thousands of aspen genes have been generated at Umeå Plant Science Centre [33]. Sixteen genes were selected from different gene mining programs, that showed increased growth (height, diameter, or both) in the greenhouse and over several independent transformation events. The gene mining programs were not targeted towards genes with a known or predicted function, but those selected were either predicted to code for a transcription factor or were shown in a set of microarray experiments [34] to have expression in the wood-forming zone. Therefore, this was a random screen of up- or down-regulation of over 1000 genes selecting for increased growth, at least in terms of other phenotypes than growth under greenhouse conditions. Three independent lines/transformation events for each of the 16 genes were selected, and the 3 lines with a given gene construct are hereafter called one “transgenotype”. For the purpose of this study, the identified and the predicted functions of the genes are unimportant, but a description is provided (Suppl. Table S1).

Halland field sites

All genotypes, specifically the 12 SwAsp accessions, 48 (16 genes x 3 events) transgenics, and wild type (WT) hybrid aspen (the background *Populus tremula x tremuloides* clone ‘T89’) were propagated using *in vitro* culture, grown in the greenhouse, and planted in randomised block designs in Halland, with one site at Arlösa and one site at Våxtorp (Supplementary material, Methods).

Experimental design

Initial tests of the effect of field block were conducted for each trial. Consistency among transformation events (lines) of each transgenic construct (each transgenotype) was then tested for each construct at each site. Each transgenotype was compared with the wild type (WT) hybrid aspen clone ‘T89’ for the target traits height and stem diameter in each transgenic trial. To examine the magnitude of the target phenotype in transgenotypes compared to natural variation in the SwAsp collection, the coefficient of variation (standard deviation / mean) in tree height was used to evaluate variability between trials and populations by examining values for the following groups: the transgenotypes at Våxtorp, transgenotypes at Arlösa, “SwAsp 12” accessions at Arlösa, the same “SwAsp 12” accessions at the Ekebo common garden 90 km away, and the ten “SwAsp Halland” accessions (originating from Simlångsdalen, Halland, local to Arlösa) growing at Ekebo. The performance of each transgenotype was further compared with ‘T89’ for the non-target phenotypes bud set phenology, biotic stresses (see Phenotyping

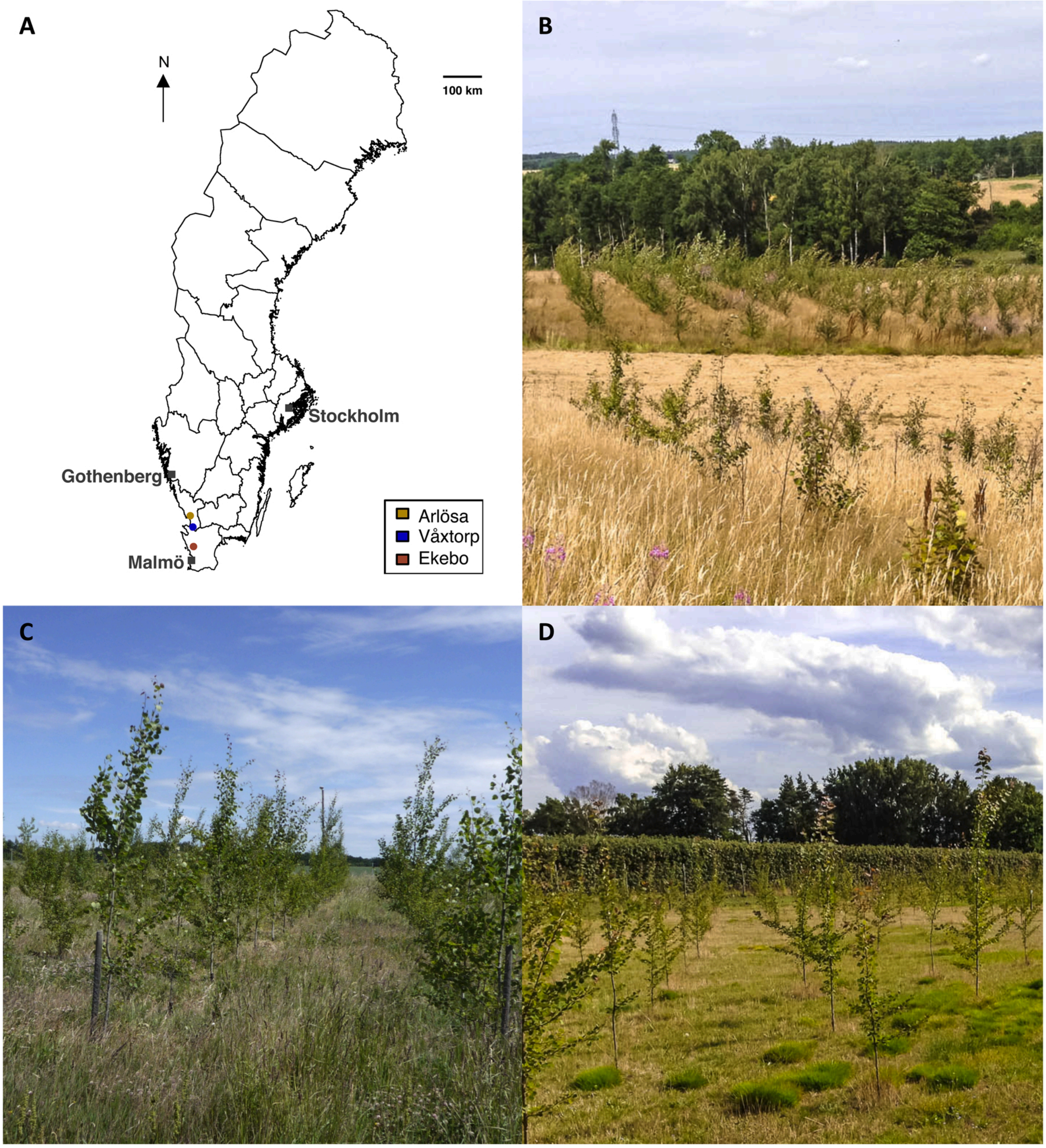

Fig. 1. Map of Sweden showing the location of the field trials **(A)**. Photographs of the field trials at **(B)** Arlösa, Halland, containing 12 accessions from the Swedish Aspen (SwAsp) collection (foreground) and GMO hybrid aspens (background), **(C)** the SwAsp collection at Ekebo, Skåne, and **(D)** the trial of GMO hybrid aspen at Våxtorp, Halland.

Table 1
Geographical and climatic characteristics and field trial design parameters for sites in this study. Sites comprise of trials of genetically modified hybrid aspen (GMO), Swedish aspen collection (SwAsp) with natural aspen accessions, or both GMO and SwAsp. The GMO trials additionally included the wild-type hybrid aspen clone 'T89' used as the construct background. Details of the genotypes are provided in Supplementary material Table S1 (GMO) and Table S2 (SwAsp).

Site	Latitude	Longitude	Elevation (m)	Mean annual temperature (°C)	Annual precipitation (mm)	Trial	*n* blocks	*n* (trans)genotypes
Våxtorp	56.4209	13.0777	39	7.5	775	GMO	10	16
Arlösa	56.6971	12.9403	18	7.6	799	GMO	10	16
						SwAsp	3	12
Ekebo	55.9435	13.1087	76	7.5	699	SwAsp	4	22[§]

[§] Although the Ekebo common garden includes 113 aspen genotypes collected from the whole of Sweden, this study considered only data from 12 SwAsp accessions drawn from the whole of Sweden (one from each of 12 sub-populations) and ten accessions originating from Simlångsdalen, Halland, ca 15 km from the Arlösa GMO trial.

section below) and initiation of flower buds. To assess environmental variability of phenotypes, 3 traits were compared between the transgenic trials at Våxtorp and Arlösa to observe how much variation might be expected in the same transgenotype between sites of slightly different bioclimatic characteristics located 35 km apart.

Phenotyping

Stem height and stem diameter (ca 3 cm above ground) were measured in autumn of the establishment year (2010), and after 2 (2012) and 6 years (2016) in all trees at Arlösa and Våxtorp and after 2 (2006) and 6 (2010) years in the SwAsp collection at Ekebo. Shoot productivity was measured as shoot number in 2012, as the number of stems sprouting from the base of the tree in addition to the original stem. Bud set was scored in September 2012 on a scale of 0–3 where 3 is a closed bud [31]. Flower buds were classified as present or absent from inspections of all trees in February and March 2017. Foliar rust infection by *Melampsora* spp. was scored on a scale of 0–3 where 3 is severe. Damage to foliage by arthropod herbivores (Suppl. Table S3) was scored from 0–3 where 3 is severe [35]. Counts of arthropod herbivore morphospecies on each tree were used to calculate the Simpson index of diversity, 1-D [36]. Further details of phenotyping are provided (Supplementary material, Methods).

Statistical analyses

Statistical analyses were conducted in the R statistical computing environment [37]. First, one-way analysis of variance (ANOVA) was used to evaluate block effects in the transgenotype trials at Våxtorp and Arlösa with phenotype as the dependent variable and block as the independent variable. A Tukey test was employed to examine block-wise significant effects. To test for differences among transformation effects (lines) within a construct, differences among genotypes for intentional and unintentional phenotypes, and differences in phenotypes between sites, mixed effects linear models were run in R using the lme4 [38] and lmerTest [39] packages. Denominator degrees of freedom were estimated with the Satterthwaite approximation [40]. Differences among lines of each construct were tested for after two growing seasons (2012), by comparing the variation in tree height among the three independent transformation events for each gene construct separately using individual linear mixed models with block as a fixed independent variable and line, a random effect, nested in construct, a random effect. Type III analysis of variance was conducted on F ratios and significance of random effects was estimated by backward elimination of the random terms from the model. Due to the number of tests conducted (one test per genotype) *P*-values were considered that were adjusted using the Holm-Bonferroni method [41] applied with the p.adjust function in R. Differences among genotypes for intentional and unintentional phenotypes in each transgenic trial separately were tested using a model with block as a fixed independent variable and construct, a random effect. Significant differences from 'T89' were determined using a Tukey test. Differences in transgenotypes between sites were compared for stem height using a one-way ANOVA with site as the independent variable, and for the count data shoot number and bud set score Kruskal-Wallis tests were conducted with site as the independent variable, using the kruskal.test function in R. Arthropod community composition differences between the transgenotype collection and the SwAsp collection in Arlösa were evaluated using permutational MANOVA (PERMANOVA) as in [35] (Supplementary material Methods). The counts of present or absent flower buds of each transgenotype in the Våxtorp trial in spring 2017 were compared to the expected ratio of present and absent of flower buds in 'T89' using a Fisher's exact test using the fisher.test function in R. Likewise, a Fisher's exact test was used to compare those trees that had set bud versus those that had not. Significance was determined at $P < 0.05$.

Results

Effects were consistent between transformation events

Comparisons of transgenotypes were conducted in Våxtorp and Arlösa to assess the variation in height in spring 2012 (after possible establishment effects). The lines within a transgenotype showed, overall, consistent results (Suppl. Fig. S1 and S2). While transgenotypes differed significantly in height (Suppl. Tables S4 and S5), lines within each transgenotype did not differ ($P < 0.05$) with the exception of TFSTT051, which showed differences among lines ($P < 0.05$) but not after adjustment for multiple testing (Holm method, $P_{\text{adj}} = 0.6372$). Studies have indicated that individual lines generated from transformation of the same gene construct could vary, for example due to position effects and/or genetic differences resulting from the tissue culture procedure [42]. In this case, outliers had however already been removed during the selection process as the goal was to identify genes whose changed expression consistently could affect growth of the tree.

Several transgenotypes exhibited differences in growth

The differences in stem height and diameter (as proxies of biomass productivity) were compared between each of the 16 transgenotypes and the 'T89' wild-type clone after 2 (Suppl. Tables S4 and S5) and 6 growing seasons. Stem growth and yield were considered to be intentional, target effects of the genetic modification and there were clear differences among transgenotypes, but at the time of measurement in autumn 2016 none of the transgenotypes had a significantly higher productivity in terms of stem height or diameter than the wild-type in Våxtorp (Fig. 2). In Våxtorp (Fig. 2) 3 and in Arlösa (Suppl. Fig. S3) 2 transgenotypes were smaller in height than the WT. Furthermore, a low survival rate was noted of one genotype, AtGA20ox1, which had only one remaining replicate in each trial after two growing seasons in the field and no surviving replicates after three growing seasons.

The variation in the target trait in the different transgenotypes was compared to that in the natural aspen population. The different classes of trees (natural accessions *vs*. transgenics and site *vs*. site) were of different average sizes, as the hybrid aspen T89, the genetic background for the transformations, grew more quickly than the natural accessions under these environmental conditions; the average size was ca. 200 cm for the transgenotypes compared to <100 cm for the SwAsp accessions. Therefore, the coefficient of variance (CV) (SD/Mean) was calculated to compensate for these size differences when comparing the magnitude of variation. The CV of the collection of transgenic lines in the two sites was as expected similar (Fig. 3), and the CV between the collection of transgenic lines and the collection of "SwAsp 12" set grown at the same site (Arlösa) was also similar. The CV of the "SwAsp12" set grown in Ekebo was higher than in Arlösa, highlighting the differences in variation around the mean in a natural population grown in different environments. The variation in height in the subpopulation of SwAsp sampled in Halland was, as previously reported [25] considerably smaller than that in the whole SwAsp population (Fig. 3). Taken together, this observation indicates that when transgenics, selected for enhanced growth under greenhouse conditions, were grown under natural conditions, the majority did not grow significantly better than wild type plants and several of them had decreased growth instead. This is not surprising given the fact that field conditions are considerably different and much more variable than controlled conditions. Furthermore, field conditions provide new challenges to growth that might not be present under controlled conditions. This illustrates that field experiments are crucial to select most well-performing genotypes, whether transgenic or not. Moreover, variation in the target trait (growth) among the transgenics was similar to the variation in the same trait in the natural population (sampled throughout whole Sweden), although larger compared to the local natural population.

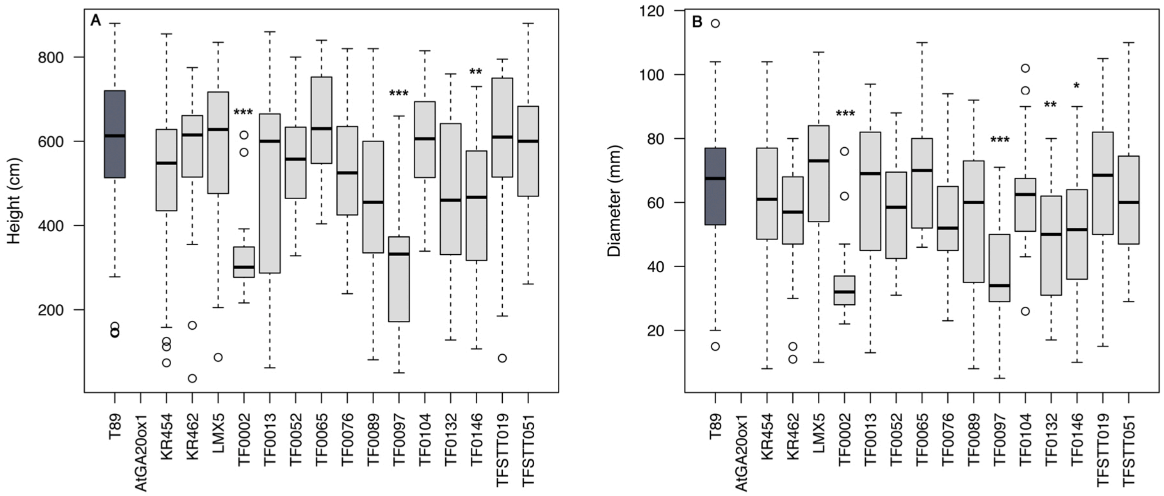

Fig. 2. Target phenotypic effects were tested by comparison of transgenotypes, (light grey boxes) against the wild-type ‘T89’ (dark grey boxes). Target traits **(A)** stem height (ANOVA $F_{(15,\ 425)} = 7.042$, $P < 0.001$) and **(B)** stem diameter (ANOVA $F_{(15,\ 427)} = 6.317$, $P < 0.001$) measured in the Våxtorp trial in autumn 2016 (prior to harvest) are shown. Statistical significance of a Tukey Test comparison between a transgenotype and ‘T89’ is indicated at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). There were no surviving individuals of line AtGA20ox1. For the same comparisons in Arlösa, see Supplementary material Figure S1.

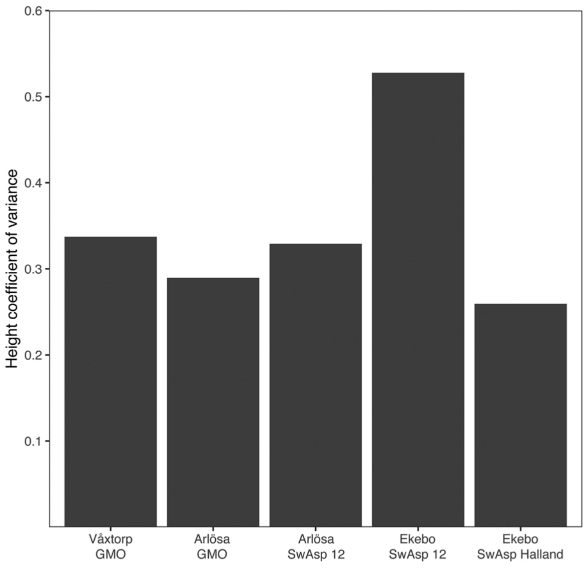

Fig. 3. Comparison of the coefficient of variation of tree height at age two years among the transgenic trials at Våxtorp and Arlösa, the SwAsp 12 genotypes at Arlösa, the SwAsp 12 genotypes at the Ekebo common garden, and the ten SwAsp genotypes from Halland (collected close to Arlösa) at the Ekebo common garden.

Phenotypic variation in biotic interaction traits was smaller than natural variation

Unintentional effects were regarded as differences between the transformants and the wild-type for non-target phenotypes. When material has been selected for increased growth under greenhouse conditions, such non-target phenotypes may include environmentally influenced traits such as susceptibility to biotic or abiotic stresses, plant architecture or reproductive development. It is important to assess unintentional effects from a purely academic perspective but also in accordance with regulatory requirements, especially in the case of flowering or vegetative propagation that is considered a potential contamination risk. Here, variation was measured in some of the most ecologically relevant non-target traits, namely the number of vegetative shoots arising from each planted stem, timing of bud set, the infection by rust fungus of the genus *Melampsora* and the community of arthropod herbivores in the canopy.

The progression of autumn growth arrest (a score of bud set development), which in aspen is triggered by a shortened photoperiod, was a target trait for the AtGA20Ox1 transgenotype which had been chosen for consistently late bud set. However, since replicates of AtGA20Ox1did not survive in the field and no other transgenotypes had prior reported changes in phenological characteristics, bud set was, in the other transgenotypes, regarded as a non-target trait.

An ANOVA analysis (Table 2, Suppl. Table S6) showed that variation in transgenotypes and ‘T89’ differed depending on the phenotype and the site at which it was scored. While median height was greater in Arlösa, the slightly warmer site with higher precipitation than Våxtorp, comparisons within lines between sites revealed significant differences only in the two lines, TF0002 and TF0097 (Fig. 4A). The non-target trait, number of shoots varied little in Våxtorp (Table 2) ranging from 0 to 6 extra shoots per tree with a median of 0, whereas in Arlösa the number of shoots ranged from 0 to 20 (Suppl. Fig. S4) with median 1.8 (Fig. 4 B). Only in Arlösa was there a significant difference in shoot number between transgenotypes (Table 2). At both sites there was a significant effect of field block on shoot number (Suppl. Table S6), indicating an environmental component of variation in this non-target trait. Timing of bud set was also different among transgenotypes at both sites (Fig. 4C), to a greater degree at Arlösa than Våxtorp (Table 2). When foliar rust infection was quantified, ANOVA indicated significant differences between the transgenotypes, both at the Arlösa and Våxtorp sites, hence the change in the expression of genes assumed to affect growth could have direct or indirect effects on the trees’ capacity to withstand rust infection. In contrast, no differences in overall herbivore damage were found between the transgenotypes, nor could any significant difference be detected in the arthropod herbivore diversity (as indicated by the Simpson index) at either Våxtorp or Arlösa (Table 2).

Another aim was to relate variation in ecologically important non-target traits, resulting from uncontrollable processes in the generation of transgenics, to the extent of variation in the natural population. No

Table 2
Genotypic (random) effects of non-target phenotypes between transgenotypes in Arlösa and Våxtorp. For each phenotype in each trial, a linear mixed model ANOVA was employed with Block as a fixed factor, transgenotype as a random factor, and phenotype as the response variable. The result of single term deletion of the random effect Line is provided for each transgenotype (to test for significance of the reduction in the residual sum of squares of model 1 with the random effect dropped, compared to model 2 with the random effect included). logLik = log-likelihood for the model, AIC = model AIC calculated as -2*(logLik - n); LRT = likelihood ratio test statistic; Df = degrees of freedom for the LRT statistic; *P*-value = *P*-value of likelihood ratio test. Fixed effects are detailed in Supplementary material Table S6.

Phenotype	Model	Våxtorp						Arlösa					
		n	logLik	AIC	LRT	Df	*P*-value	n	logLik	AIC	LRT	Df	*P*-value
Number of shoots	1	12	−569.5	1163				12	−1054.1	2132.3			
	2	11	−570.2	1162.4	1.4023	1	0.2363	11	−1056.9	2135.9	5.5763	1	**0.0182**
Bud set	1	12	−322.36	668.73				12	−380.6	785.19			
	2	11	−325.4	672.79	6.064	1	**0.0138**	11	−388.48	798.97	15.777	1	**< 0.0001**
Foliar rust infection	1	12	−347.73	719.46				12	−257.93	539.86			
	2	11	−358.18	738.35	20.897	1	**< 0.0001**	11	−264.25	550.51	12.644	1	**0.0004**
Arthropod herbivore damage	1	12	−112.78	249.56				12	−108.63	241.26			
	2	11	−112.78	247.56	4.01E-12	1	1	11	−109.71	241.42	2.1555	1	0.1421
Simpson index	1	12	2004	−3984				12	1027.6	−2031.3			
	2	11	2003.3	−3984.6	1.307	1	0.2529	11	1027.6	−2033.3	4.55E-13	1	1

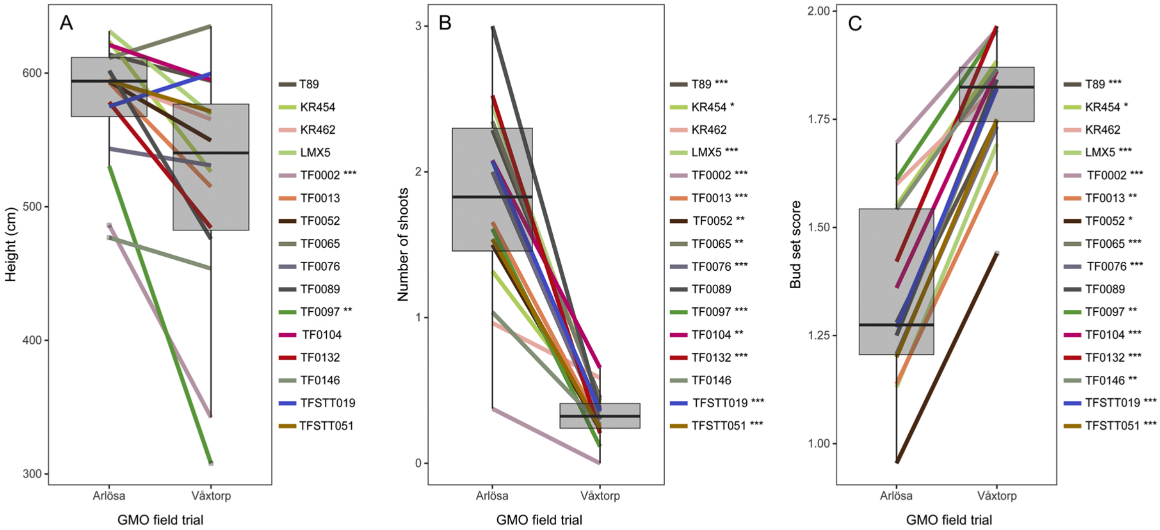

Fig. 4. Comparisons of (A) tree height, (B) shoot productivity, and (C) bud set score between the transgenic trials at Arlösa and Våxtorp. Boxes show the overall population data distribution for each trait. Grey lines represent the wild type 'T89' and coloured lines represent the transgenotypes indicated in the legend, which connect the median value for each genotype at each site. Where present, significant differences in a genotype between the two gardens are indicated in the legend as *** $P < 0.001$, ** $P < 0.01$ and $P < 0.05$.

trees in the collection of natural accessions had additional shoots, so the variation in this trait could not be compared. However, traits relating to biotic interactions were scored in Arlösa in parallel between the "SwAsp 12" genotypes and the transgenotypes and could be directly compared. Despite the considerably smaller height of the SwAsp genotypes than the transgenotypes (Fig. 5A), the variation in the Simpson index of herbivore diversity had an equal range, but a greater dispersion of values in the "SwAsp12" (Fig. 5B), despite no significant difference in the medians. The overall scores of the SwAsp accessions were also significantly greater than those of the transgenotypes ($P < 0.001$). A permutational MANOVA indicated that the arthropod herbivore communities between the transgenotypes and the natural accessions did not differ after correction for tree height (Table 3). This is important since herbivore communities could have been responding to plant size. The foliar rust infection score differed significantly between the transgenotypes; as illustrated by the box plot, most transgenotypes had a median score of 0, while one outlier genotype had score 1 and one had score 2 (Fig. 5C). In contrast, and consistent with previous results from earlier studies of the SwAsp population [26,35], rust was more prevalent in SwAsp, with a median score in SwAsp 1 and distribution of scores between 0 and 3. Taken together, although we could detect some variations in non-target traits as a result of the transformations, this variation was smaller than the natural variation in the same trait.

Variation in bud set in the SwAsp collection is very considerable, in fact so large that bud set scoring is almost not meaningful in Ekebo as the critical photoperiod for bud set for genotypes from northern Sweden is longer than the day length at midsummer [43]. While bud set is tightly coupled with latitude due to its relationship with day length and its timing is consistent within a genotype among years, it varies greatly in the SwAsp collection according to the different sampling latitudes [25,31], so this vast variation was compared with the transgenotypes in a hybrid aspen background with caution. When bud set was scored at a given date (week 37) in both GMO trials, the proportion of trees that had set bud did not differ from those in the whole SwAsp population at Ekebo in the years 2005 and 2006 when the trees were at comparable developmental stages to the GMO trees when bud set was scored (Fig. 6). Despite this showing that bud set was not later, and therefore growing season length was not longer, in the GMO trials than in SwAsp, a

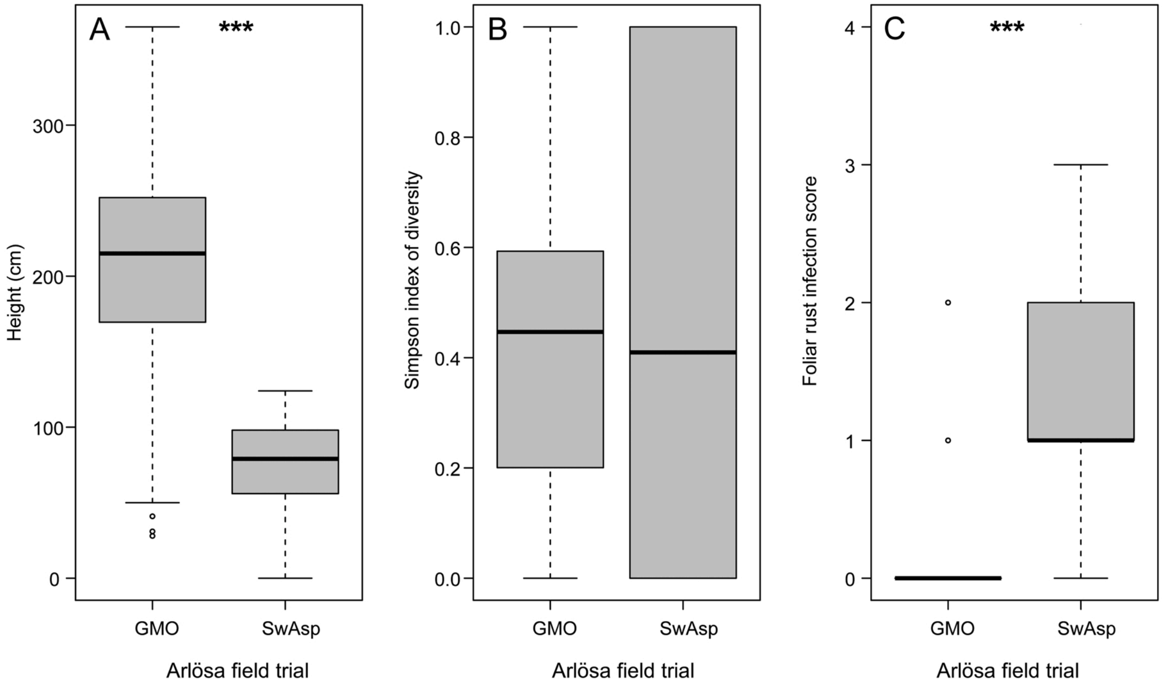

Fig. 5. Comparisons between the transgenic and SwAsp12 collections at Arlösa for (A) stem height, (B) Simpson index of arthropod diversity, and (C) foliar rust infection intensity in summer 2012. Differences between the collections, where significant, are denoted by *** at $P < 0.001$.

Table 3

Permutational MANOVA comparing the Bray-Curtis dissimilarity matrices of arthropod herbivore morphospecies between the collections of transgenotypes and SwAsp12 genotypes (Collection, a fixed factor) with tree height as a covariate. *F* ratio is the test pseudo-F ratio. *P*-value is based on 1000 permutations of the data matrix.

	DF	SS	R^2	*F* ratio	*P*-value
Height	1	9.646	0.15767	40.1514	**0.001**
Collection	1	0.12	0.00197	0.5011	0.757
Residual	214	51.414	0.84036		
Total	216	61.181	1		

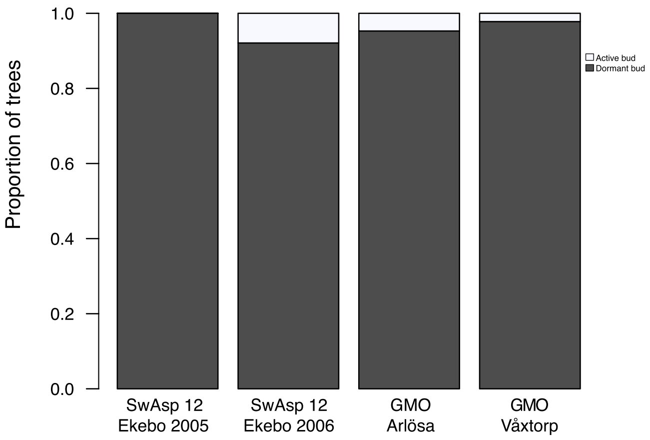

Fig. 6. Proportion of trees that with an active (pale grey shading) or dormant (dark grey shading) bud scored in week 37 at age two and three years, in 2005 and 2006 respectively, in the "SwAsp 12" genotypes the Ekebo common garden and in 2012 for two-year old trees in the transgenic collections at Arlösa and Våxtorp.

comparison of the height of two-year-old stems between the GMO and "SwAsp 12" accessions in Arlösa indicated a clear difference, with median values of height of transgenics more than two-fold higher than SwAsp (ANOVA: $F_{(1,481)} = 598.2$, $P < 0.001$, Fig. 5A), indicating a vigour unique to the T89 genotype and the transgenics based on it.

Flower bud formation was not affected

Aspens flower in early spring, before leaf flush, from flower buds formed during the previous growing season. Although little is known about the factors triggering the transition from juvenility to maturity in aspen, i.e. the age when the first flowers appear, typically in the Swedish climate, flowering is uncommon in aspens younger than 20 years. However, unexpectedly severe drought in the summer of 2016 resulted in a transition to reproductive growth in many trees, so that in late winter/early spring of 2017, flower buds were observed in 104 trees at Våxtorp (Table 4), while in Arlösa only one tree had flower buds. The conditions for our transgenic trial stipulate that all trees of genotypes that formed flower buds should be harvested, so the Våxtorp trial was terminated after final phenotype scorings had been performed. This unexpected flowering made it possible to evaluate whether the propensity to flower and spread pollen or seeds was unintentionally increased by the transformation. The frequency of flowering trees was in no transgenotype larger than the frequency in 'T89' (Table 4). However, a correlation was found between size and transition of flowering; larger trees had on average more flower buds (Fig. 7; ANOVA $F_{(1,\ 445)} = 22.83$, $P < 0.0001$).

Discussion

In this study quantitative data was generated on factors with relevance for future evaluations of risk when trees, improved by biotechnological means, should be grown in the natural environment, either in field experiments or commercially after a market release. Transgenic trees generated in the laboratory were used, with the intention to hold

Table 4

Proportions of trees with flower buds in the GMO trial of hybrid aspens in Våxtorp, Halland in spring 2017. Proportions of flowering individuals were compared between the wild-type (T89) and each transgenotype (construct). The odds ratio and *P-value* of a Fisher's exact test are reported. The result significant at $P < 0.05$ is in bold face.

Genotype	Number of non-flowering trees	Number of trees bearing flower buds	Proportion of trees bearing flower buds	Odds ratio	P-value
T89	64	28	0.30	–	–
KR454	18	5	0.22	1.57	0.607
KR462	23	6	0.21	1.67	0.353
LMX5	18	7	0.28	1.12	1.000
TF0002	15	3	0.17	2.17	0.390
TF0013	21	5	0.19	1.83	0.328
TF0052	17	3	0.15	2.46	0.269
TF0065	15	9	0.38	0.73	0.624
TF0076	23	7	0.23	1.43	0.497
TF0089	15	4	0.21	1.63	0.580
TF0097	13	2	0.13	2.82	0.225
TF0104	26	3	0.10	3.76	**0.031**
TF0132	21	4	0.16	2.28	0.207
TF0146	21	6	0.22	1.53	0.475
TFSTT019	21	5	0.19	1.83	0.328
TFSTT051	19	7	0.27	1.19	0.812

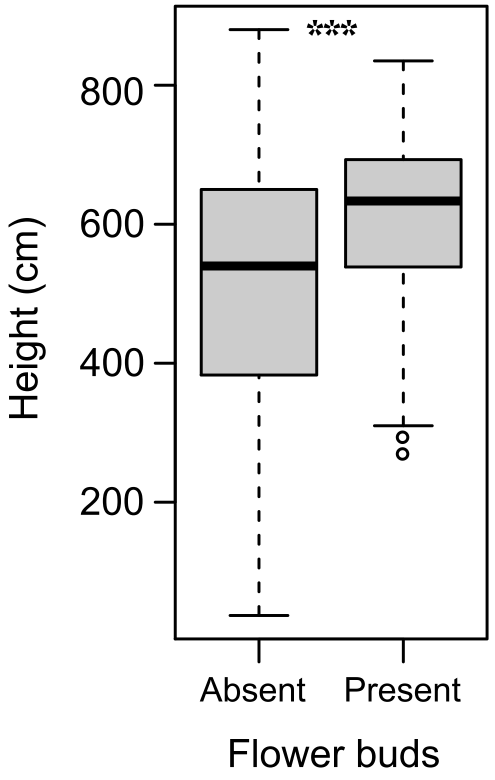

Fig. 7. Tree height in autumn 2016 in all individuals in the transgenic field trial at Våxtorp partitioned by flowering incidence (presence or absence of flower buds) the subsequent spring. A significant height difference between trees bearing flower buds and trees without flower buds is indicated (***), ANOVA ($F_{1,\ 445} = 22.83$, $P < 0.0001$).

promise for commercial introduction, as they exhibited increased growth under controlled greenhouse conditions, to make it as similar as possible to what researchers, companies and regulatory authorities may encounter.

First, the data illustrate that well replicated field experiments with a randomized design are necessary as new genotypes are to be introduced to the market. This comes as no surprise for researchers and breeders, but the current regulatory regime that makes field experiments with transgenic plants and trees in some countries expensive and complicated, in others impossible, is therefore a major hurdle for the use of plant biotechnology in society [1]. Growth is perhaps the most complex trait of all [1] and it is not unexpected that only a small fraction of genotypes with improved growth under greenhouse conditions also show an enhancement in the field. It is likely that genetically "simpler" traits like wood quality or herbivore resistance would show a better correspondence between laboratory and field conditions – in other words that expression of single genes could give an effect across many environments. Nevertheless, field experiments are necessary and the data also illustrate how differences in the local environment require an experimental design with sufficient replicates and that could take block effects/spatial differences from consideration.

Secondly, it is shown that non-target traits could be affected. Again, this is unsurprising as the trees studied were preselected to be affected in one important trait – growth under controlled conditions – which potentially could lead to other effects. In other studies of forest tree species, genetic modifications have had no [44] or mild effects on non-target traits, with the latter being on only one of several transgenic clones studied [45,46]. Furthermore, it is worth considering how non-target traits are defined; for example in the case of transgenic *Populus* hybrids modified for cell wall acetylation, it could be foreseen that other compounds present in cell walls (e.g. condensed tannins or salicinoids) may be affected, which in turn have known effects on herbivores [47]. Irrespective of context, a mild amount of unintended variation may be expected, for example through phenotypic plasticity, as observed in traditionally bred plants [48]. If random transgenics had been chosen without pre-selection, the average effect on non-target traits would likely have been smaller than the effects that were observed. However, the third and most important finding is that the variation in non-target traits that were measured was in all cases similar to or smaller than the variation in the same traits in the natural population. Greater variation was not detected in growth between transgenotypes than between natural accessions, either in propensity for vegetative or sexual reproduction, and the variation in interaction with pathogens and herbivores was significantly smaller among transgenotypes. Of course, with more than 16 transgenotypes/48 transgenic events, it would have been likely that trees would be found with, e.g., much increased or decreased resistance to a specific pathogen, but the likelihood of finding even more varying trees among the natural accessions is higher.

This has implications on how a scientifically relevant regulatory system for transgenic plants should be constructed. If the variation in non-target traits is smaller than the natural variation among plant individuals (which is considered not to be risky enough to justify regulation) there is no scientific justification for considering non-target effects risky in the evaluation process. It should be stressed that the traits that were compared are those that regulatory procedures focus their attention on: growth, interaction with pathogens and herbivores and capacity for vegetative and sexual reproduction [16]. Since many controversies in the field relate to possible effects on non-target traits [49,50], questions asked in the regulatory process could include whether the crop is safe for human consumption, or if its growth has any negative effect on bees. At least where forest trees are concerned, the evidence here suggests that variation in ecologically important non-target traits is not relevant and only the effects on the target trait needs to be considered.

Conclusion

Variation has been systematically compared in non-target traits of biological/ecological importance, which could be the results of genetic modification of hybrid aspens for improved growth, and it was found that the effects were smaller than, or similar to, the natural variation in the same traits.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2021.05.005.

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